Supplemental data

Probe syntheses

General methods

2-(*R*)-2-(4,7,10-tris-tert-butylcarboxymethyl-1,4,7,10-tetraazacyclododec-1-yl)-pentanedioic acid-1-tert-butylester was obtained as described previously (31). All other reactants and reagents were of commercial grade and were used without further purification.

NMR: NMR spectra were recorded on a Varian 500 NMR system equipped with a 5 mm broadband probe (^{1}H NMR: 499.81 MHz, ^{13}C: 125.68 MHz). ^{13}C spectra in D_2O were referenced using a MeOD spike.

Preparative HPLC: Purifications were performed using Method 1: Column: Phenomenex LUNA C18(2) 10 µm 250×21.2 mm, UV detection at 220, and 254nm, flow rate: 15 ml/min, solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in MeCN, gradient: 0-2 mins hold at 5% B, 2-24 mins gradient to 95% B, 24-25 mins hold at 95% B, 25-28 min gradient to 5% B, 28-30 mins re-equilibrate at 5%B. Method 2: Column: Restek, UltraAqueous C18, 5µm 250×10 mm, UV detection at 220nm, flow rate: 3 ml/min, solvent A: 0.1% TFA in water, B: 0.1% TFA in MeCN, gradient conditions: 0-5 mins hold at 2% B, 5-15 mins gradient to 20% B, 15-18 mins gradient to 60% B, 18-20 mins hold at 60% B, 20-22.5 mins gradient to 2% B, 22.5-25 mins re-equilibrate at 2%B.

HPLC-MS: HPLC-MS purity analysis was carried out on an Agilent 1260 system using Method A: Column: Phenomenex Luna, C18(2), 100×2 mm, flow rate: 0.8 ml/min, UV detection at 220, 254 and 280 nm, solvent A: 0.1% formic acid in water, B: 0.1% formic acid in MeCN, gradient conditions: 0-1 mins hold at 5% B, 1-9 mins gradient to 95% B, 9-10 mins hold at 95% B, 10-12 mins gradient to 5% B, 12-15 mins re-equilibrate at 5%B. Method B: Column: Restek, UltraAqueous C18, 5µm 250×4.6 mm, UV detection at 220nm, flow rate: 0.8 ml/min, solvent A: 0.1% formic acid in water, B: 0.1% formic acid in MeCN, gradient conditions: 0-2 mins hold at 5% B, 2-12 mins gradient to 50% B, 12-13 mins gradient to 95% B, 13-15 mins hold at 95% B, 15-16 mins gradient to 5% B, 16-18 mins re-equilibrate at 5%B.

HPLC-ICP-MS: HPLC-ICP-MS purity analysis was carried out on an Agilent 1260 HPLC system coupled to an Agilent 8800-QQQ ICP-MS system, using the following HPLC method: Column: Restek, UltraAqueous C18, 5µm 250×4.6 mm, UV detection at 220 nm, flow rate: 0.8 ml/min, solvent A: 0.1% TFA in water, B: 0.1% TFA in MeCN, gradient conditions: 0-2 mins hold at 5% B, 2-12 mins gradient to 50% B, 12-13 mins gradient to 95% B, 13-15 mins hold at 95% B, 15-16 mins gradient to 5% B, 16-18 mins reequilibrate at 5%B.

UV Titration: Into a 1.5 mL quartz cuvette is placed 10 µL of ligand solution and 1 mL of an arsenazo III solution (10 µM arsenazo III in 0.15 M NH_4OAc buffer pH 7). The cuvette is placed into a UV/Vis spectrophotometer and zeroed at 656 nm. Aliquots of 10 µL of a 4.85 mM $Pb(NO_3)_2$ solution (or 0.485 mM solution close to the end point), are titrated into the cuvette until a positive absorbance is observed. A positive absorbance represents the end point of the titration.

Preparation of Gd-Hyd

Scheme S1: Preparation of Gd-Hyd (2-(*R*)-2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraazacyclododec-1-yl) pentanedioic acid-1-hydrazide gadolinium complex**)** in three steps form DOTAGA.

a. *Synthesis of 2-(R)-2-(4,7,10-tris tert-butylcarboxymethyl-1,4,7,10-tetraazacyclododec-1-yl)-pentanedioic acid-1-N'-tert-butoxycarbonyl-N-hydrazide:* 2-(*R*)-2-(4,7,10-tris tert-butylcarboxymethyl-1,4,7,10-tetraazacyclododec-1-yl)-pentanedioic acid-1-tert-butylester (500 mg, 713 μmol) and O-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU, 325 mg, 856 μmol) were dissolved in dry DMF (2.5 ml). After 5 minutes, solid tert-butyl-semicarbazide (113 mg, 856 μmol) was added and stirring was continued for 24 hours. After the solvent was evaporated, the residue was purified using Method 1 to yield 574 mg (704 μmol, 98.7%) of a white solid product. The product was characterized by ^{1}H NMR (DMSO-d_6, 60°C): 9.27 (br s, 1H), 8.21 (br s, 1H), 4.15-3.15 (m, 7H), 3.07-3.14 (m, 8H), 2.90-2.93 (m, 8H), 2.31 (m, 2H), 1.87-1.95 (m, 2H), 1.46, 1.44, 1.40 (3s, 45H). ^{13}C NMR (DMSO-d_6, 60°C): 170.8, 170.6, 169.1, 167.4, 155.0, 81.9, 81.3, 81.0, 78.7, 62.7, 55.2, 54.1, 52.1, 51.7, 48.7, 46.0, 40.0, 29.8, 23.1. LC: Method A, t_R = 5.64 minute. LC/MS (ESI+): $C_{40}H_{74}N_6O_{11}$: *m/z* (%): calcd 815.5 [MH^+]; found: 815.4 (MH^+).

b. *Synthesis of 2-(R)-2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraazacyclododec-1-yl)-pentanedioic acid-1-hydrazide:* 2-(*R*)-2-(4,7,10-tris tert-butylcarboxymethyl-1,4,7,10-tetraazacyclododec-1-yl)-pentanedioic acid-1-*N'*-tert-butoxycarbonyl-*N*-hydrazide (100 mg, 123 μmol) was dissolved in a mixture of trifluoroacetic acid (TFA, 1.5 ml), triisopropyl silane (90 μl) and 1-dodecathiol (90 μl). The mixture was stirred at room temperature overnight. The volatiles were removed by vacuum. To the residue was added diethyl ether to precipitate out the product as a white solid. The diethyl ether was removed and the pellet resuspended and washed in diethyl ether three times. The residue was purified using HPLC Method 2. The lyophilized solid was re-dissolved in half-concentrated HCl. After the solution was stirred for 3 hours at room temperature, the solution was lyophilized. The residue was re-dissolved in water and the pH was adjusted to 7 using ammonium hydroxide. After lyophilization, the solid was re-dissolved in water (2.00 ml) and subjected to a UV titration using arsenazo III (see above) to determine the concentration of ligand (36 mM, 72.6 μmol, 58.5%). The product was characterized by ^{1}H NMR (D_2O, 60°C, pH9): 4.45 (m, 5H), 4.16 (m, 2H), 3.90-3.65 (m, 16H), 3.10 (t, *J* = 7.1Hz, 2H), 2.57 (br m, 2H). ^{13}C NMR (D_2O, 60°C, pH9): 170.5, 174.3, 171.1, 63.1, 56.7, 55.7, 51.2, 50.0, 47.4, 46.3, 45.2, 31.6, 25.8. LC: Method B, t_R = 5.74 minute. LC/MS (ESI+): $C_{19}H_{34}N_6O_9$: *m/z* (%): calcd 491.2 [MH^+]; found: 491.1 (MH^+).

c. *Synthesis of 2-(R)-2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraazacyclododec-1-yl)-pentanedioic acid-1-hydrazide gadolinium complex (Gd-Hyd):* The stock solution of 2-(R)-2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraazacyclododec-1-yl)-pentanedioic acid-1-hydrazide obtained above was treated with $GdCl_3{\bullet}6H_2O$ (27.0 mg, 72.6 μmol) and the pH of the solution was adjusted to 6.2. After the solution was stirred for 24 hour, the pH was adjusted to 5.5 and the solution passed through a chelex column to remove any free Gd. The pH of the solution was corrected to 7.0 and lyophilized to yield 30.0 mg (46.5 μmol, 64.1%) of the title compound. Both arsenazo III and xylenol orange test were negative demonstrating the absence of non-chelated Gd(III). Gd-Hyd was characterized by LC: Method B, t_R = 6.83 minute. LC/MS (ESI+): $C_{19}H_{30}GdN_6O_9$: *m/z* (%): calcd 645.1 [MH^+]; found: 645.2 (MH^+).

Preparation of Gd-DiMe

Scheme S2: Preparation of Gd-DiMe ((*R*)-2,2',2''-(10-(1-carboxy-4-(2,2-dimethylhydrazinyl)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid gadolinium complex) in three steps form DOTAGA

a. *Synthesis of (R)-tri-tert-butyl 2,2',2''-(10-(1-(tert-butoxy)-5-(2,2-dimethylhydrazinyl)-1,5-dioxopentan-2-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate:* (*R*)-5-(*tert*-butoxy)-5-oxo-4-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanoic acid (500 mg, 713 μmol) and N,N′-diisopropylcarbodiimide (DIC) (116 mg, 927 μmol) were dissolved in dichloromethane (25 mL). After 5 minutes, *N*,*N*-dimethylmethanediamine (70.5 μl, 927 μmol) was added and stirring continued for 24 hours . After the solvent was evaporated, the residue was purified using Method 1 to yield 350 mg (471 μmol, 66%) of a white solid product. The product was characterized by ^{1}H NMR (DMSO-d$_6$, 60°C): 9.45 (br s, 1H), 8.31 (br s, 1H), 3.77 (m, 4H), 3.52 (m, 3H), 3.12 (m, 8H), 2.93 (m, 8H), 2.52 (s, 6H), 2.13 (m, 2H), 1.88 (m, 2H), 1.42, 1.40, 1.38 (3s, 36H). ^{13}C NMR (DMSO-d$_6$, 60°C): 174.2, 171.3, 169.7, 168.2, 82.7, 82.0, 81.8, 55.8, 54.9, 52.5, 49.6, 47.9, 46,9 46.5, 30.9, 28.1, 23.7. LC: Method A, $t_R = 5.22$ minute. LC/MS (ESI+): $C_{37}H_{70}N_6O_9$: *m/z* (%): calcd 743.5 [MH$^+$]; found: 743.5 (MH$^+$).

b. *Synthesis of (R)-2,2',2''-(10-(1-carboxy-4-(2,2-dimethylhydrazinyl)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid:* (*R*)-5-(*tert*-butoxy)-5-oxo-4-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanoic acid (350 mg, 471 μmol) was dissolved in a mixture of TFA (9 ml), triisopropyl silane (200 μl), 1-dodecanethiol (200 μl), water (200 μl) and methanesulfonic acid (200 μl). The mixture was stirred at room temperature for 2 hours. LC/MS showed complete reaction. The volatiles were removed in vacuum and the residue was re-dissolved in 5 ml of 1.0M HCl. After the solution was stirred for 3 hours at room temperature, the solution was lyophilized leaving 177.2 mg (342 μmol, 73%) of white solid. The product was characterized by ^{1}H NMR (D$_2$O, 60°C, pH9): 4.47 (m, 5H), 4.20 (m, 2H), 3.95-3.61 (m, 16H), 3.05 (t, *J* = 7.2Hz, 2H), 2.61 (s, 6H), 2.52 (br m, 2H). ^{13}C NMR (D$_2$O, 60°C, pH9): 175.0, 174.0, 172.2, 171.5, 62.1, 56.5, 53.4, 51.1, 50.7, 49.4, 46.7, 46.0, 32.0, 23.1. LC/MS: Method A, $t_R = 5.72$ minute. LC/MS (ESI+): $C_{21}H_{38}N_6O_9$: *m/z* (%): calcd 519.5 [MH$^+$]; found: 519.2 (MH$^+$).

c. *Synthesis of (R)-2,2',2''-(10-(1-carboxy-4-(2,2-dimethylhydrazinyl)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid gadolinium complex (Gd-DiMe):* (*R*)-2,2',2''-(10-(1-carboxy-4-(2,2-dimethylhydrazinyl)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (50 mg, 96 μmol) was dissolved in water (10 mL) and the pH of the solution was adjusted to 7 with 0.1 N NaOH. The solution was treated with $GdCl_3 \cdot 6H_2O$ (35.0 mg, 92.2 μmol) and the pH was adjusted to 6. After the solution was stirred for 24 hour, the pH was adjusted to 5.5 and the solution passed through a chelex column to remove any free Gd. The pH of the solution was corrected to 7.0 and lyophilized to yield 30.0 mg (89.8 μmol, 93.5%) of the title compound. Both arsenazo III and xylenol orange test were negative demonstrating the absence of non-chelated Gd(III). The product was characterized by LC: Method B, $t_R = 9.21$ minute. LC/MS (ESI+): $C_{21}H_{34}GdN_6O_9$: *m/z* (%): calcd 673.2 [MH$^+$]; found: 673.0(MH$^+$).

Relaxivity and BSA binding assay

Preparation of oxidized BSA, BSA-ALD: Sodium aspartate (13 mg) was added to a solution of bovine serum albumin (BSA) (100 mg) dissolved in phosphate buffered saline (2 mL, pH 7.4, 0.25 mM), followed by the addition of a solution of ferric chloride (10 μL, 10 mM) and left to stir at room temperature overnight. A BSA protein standard without the addition of ferric chloride was run in parallel as a control. Both protein mixtures were purified on PD-10 Sephadex G25 desalting columns (GE Healthcare), eluted with PBS. Protein concentrations were assessed using the 'BCA Protein Assay Kit' (Thermo Scientific). The functionalized protein (BSA-ALD) had a concentration of 20 mg/mL, whilst the control protein (BSA) had a concentration of 18.4 mg/mL. The aldehyde concentration of each protein was estimated using a standard literature DNPH assay protocol. BSA-ALD had an aldehyde concentration of 16 nmol aldehyde/mg of protein, BSA had an aldehyde concentration on 1.2 nmol aldehyde/mg of protein.

Relaxivity measurements: Aliquots of BSA-ALD (3 mg, 150 µL) or BSA (3 mg, 163 µL) were treated for 24 h at 37 °C with either Gd-Hyd or Gd-DiMe at a range of concentrations (0.1-1.0 mM), with a total volume of 300 µL maintained for all samples. After 24h, longitudinal (T_1) relaxation measurements were recorded using a Bruker mq60 Minispec at 1.41 T and 37 °C. Longitudinal (T_1) relaxation times were measured via an inversion recovery experiment using 10 inversions of duration ranging between 0.05 x T_1 and 10 x T_1. Relaxivity (r_1) was determined from the slope of a plot of $1/T_1$ vs [Gd] for 7 concentrations of Gd(III). Solutions of Gd-Hyd and Gd-DiMe (concentration range: 0.1 mM-1.0 mM), in PBS were run in parallel as standard controls.

Protein binding measurements: Gd probe in solution was separated from BSA-bound Gd probe by ultrafiltration (5,000 Da cut-off PLCC cellulosic membrane, 30 mins, 10,000 RPM). Following separation, the concentrated protein residue was diluted to a total volume of 300 µL in PBS and longitudinal (T_1) relaxation measurements of the protein bound and free-solution fractions measured. Quantification of the Gd concentration in each fraction was then determined using an Agilent 8800 ICP-MS system.

Borohydride reduction: Aliquots of BSA-ALD (3 mg, 150 µL) was treated for 24 h at 37 °C with Gd-Hyd at a range of concentrations (0.1-1.0 mM), with a total volume of 300 µl maintained for all samples. After 24h sodium cyanoborohydride (10 mg) was added to each sample to reduce any hydrazone functionality and irreversibly bind the probe to the protein. After a further 2h incubation at 37 °C, longitudinal (T_1) relaxation measurements were recorded using a Bruker mq60 Minispec at 1.41 T and 37 °C. Relaxivity (r_1) was determined from the slope of a plot of $1/T_1$ vs [Gd]. Protein-bound and protein-free solutions of Gd-Hyd were separated by ultrafiltration and the concentration of Gd bound to protein determined using an Agilent 8800 ICP-MS system.

Aorta Binding Assay

Allysine rich elastin from porcine aorta was purified as previously described (33). Briefly, after removing peripheral connective tissue and lipids, porcine aorta (5 g) was cut into small segments and suspended in 1M NaCl (50 mL) for 24h. The supernatant was discarded and the tissue pellet resuspended in 1M NaCl for 24h. The extraction process was repeated three times. The remaining tissue was washed in distilled water and defatted with chloroform:methanol (2:1) (200 mL) for 48h. The tissue was then washed with ethanol and ether and then dried in vacuo.

Stock solutions of Gd-Hyd and Gd-DiMe were prepared at a range of concentrations (0.1-1.0 mM, 10 mM PBS, pH 7.4), with EuDOTA included at a fixed concentration of 1.0 mM as an internal standard. Portions (25 mg) of lyophilized porcine aorta were then treated for 24h at 37 °C with 500 µL of either Gd-Hyd or Gd-DiMe in sealed vials. The concentration of Gd and Eu in solution was quantified before and after incubation with aorta by ICP-MS analysis and the difference in probe concentrations used to calculate the amount of Gd bound to aorta after correction for the amount of non- specific chelate binding, as estimated by the amount of EuDOTA association with aorta.

Transmetallation

Transmetallation stability studies were performed using an adaptation of the previously published protocol by Laurent et al (34). To a solution containing a Gd complex (Gd-DTPA or Gd-Hyd) was added $Zn(OTf)_2$

solution and phosphate buffer (pH 7), affording a solution with 1.0 mM Gd (in total), 1.0 mM Zn, and 67.0 mM phosphate (pH 7.0). The mixture was shaken for 6000 min at 37 °C. At regular time intervals, the longitudinal relaxation rate ($R_1 = 1/T_1$) of the reaction mixture was measured at 60 MHz. The R_1 value was standardized by measurements made in the absence of $\mathrm{Zn^{II}}$. The remaining ratio (%) of the Gd complexes in the reaction mixture was estimated by eq S1,

$$\%\text{Remaining} = [(R_1 - R_1{}^{\circ})/(R_1{}^{\text{init}} - R_1{}^{\circ})] \times 100\% \qquad \text{(S1)}$$

where $R_1{}^{\circ}$ is the R_1 for buffer solution (which did not contain the Gd complexes), 0.5 s^{-1}; $R_1{}^{\text{init}}$: R_1 at $t = 0$ hr in the presence of Gd complexes. 9.4 s^{-1} for Gd-DTPA and 10.6 s^{-1} for Gd-Hyd.

The arbitrarily defined kinetic index is given as the time required for $R_1(t)$ to reach 80% of the initial $R_1(0)$ value.

Plasma Stability

A 100 µL aliquot of the Gd-Hyd (0.01 mM) was added to 400 µL of rat plasma (Lampire Biological Laboratories). The solution was then incubated for 72 h at 37 °C. Aliquots (50 µL) were removed at specific time points, diluted with 200 µL HEPES buffer, and filtered using a 0.2 µm filter. A 5 µL aliquot of this filtrate was analyzed for transchelation using HPLC-ICP-MS (See **General methods**).

Animal models

Animal protocol: All experiments and procedures were performed in accordance with the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals" and were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Lung fibrosis model: C57Bl/6 adult male mice at 8 weeks of age (Charles River Laboratories, Wilmington MA) received a single dose of bleomycin (Fresenius Kabi, Lake Zurich, Il), prepared in sterile PBS. Bleomycin was intratracheal injected at either 1 or 2.5 units/kg body weight (50 µL total volume). Images with Gd-Hyd or the control Gd-DiMe were carried out after 1 week (early disease state), after 2 weeks (advance disease state), or after 4 weeks (fibroelastic scarring). Sham mice received equal volume of vehicle only (PBS).

To evaluate Gd-Hyd sensitivity to detecting treatment response, β-aminopropionitrile (BAPN), a pan-LOX inhibitor, was prepared in PBS, and dosed at 100 mg/kg. Starting on the day of bleomycin instillation, each mouse was dosed daily with BAPN via IP injection. Controls similarly underwent bleomycin instillation, and injected daily with PBS. Gd-Hyd MRI was performed after 2 weeks.

A total of 87 C57Bl6 mice were included to study pulmonary fibrosis:

A. Intratracheal bleomycin instillation (2.5 units/kg), imaged after 1 week (n=9), all were imaged with Gd-Hyd.
B. Intratracheal bleomycin instillation (2.5 units/kg), imaged after 2 weeks (n=31), 16 were imaged with Gd-Hyd, 9 were also imaged with Gd-DiMe for direct comparisons.
C. Intratracheal PBS instillation as Sham control (n=18), 15 were imaged with Gd-Hyd, 3 were imaged with Gd-DiMe.
D. Intratracheal bleomycin instillation (2.5 units/kg), with daily I.P. BAPN (n=12). 7 were imaged at 2 week with Gd-Hyd, an additional 5 underwent only tissue analysis.
E. Intratracheal bleomycin instillation (1 units/kg), sacrificed after 1 week (n=5) for tissue analysis.

F. Intratracheal bleomycin instillation (1 units/kg), imaged with Gd-Hyd after 2 weeks (n=8), 3 of which were serially imaged after 4 weeks.
G. Intratracheal bleomycin instillation (1 units/kg), imaged with Gd-Hyd after 4 weeks (n=4).

All animals were sacrificed after the imaging for biochemical analysis and histological evaluations.

Liver fibrosis model: Male A/J mice (Charles River Laboratories, Wilmington MA) were treated with carbon tetrachloride (CCl_4, 0.1 ml of 40% solution in olive oil) for 6, 12, and 18 weeks by oral gavage, 2-3 times per week. Control mice were fed vehicle (olive oil) only.

A total of 66 A/J mice were included to study hepatic fibrosis:

A. CCl_4 for 6 weeks (n=19), all were imaged with Gd-Hyd, 7 were allowed to recover for 6 weeks and imaged again with Gd-Hyd.
B. CCl_4 for 12 week (n=13), 10 were imaged with Gd-Hyd, 3 were imaged with Gd-DiMe.
C. CCl_4 for 18 week (n=10), all were imaged with Gd-Hyd.
D. Vehicle treated control (n=24), 12 were imaged with Gd-Hyd, 3 were imaged with Gd-DiMe, 9 underwent tissue analysis only.

All animals were sacrificed after the imaging for biochemical analysis and histological evaluations.

Magnetic Resonance Imaging

At the time of imaging, pre-injection baseline images as well as post-injection images were acquired for comparison. Probes (Gd-Hyd or Gd-DiMe) were administrated via intravenous bolus injection at 100 nmol Gd/ g body weight. Each injected probe concentration was determined by ICP-MS.

Mice were imaged on a 4.7 Tesla MRI scanner (Bruker, Billerica MA) using a custom-built volume coil. Animals were anesthetized with 1–2% isoflurane and air/oxygen mixture to maintain constant respiration rate, kept warm by a thermal pad, and monitored by a small animal physiological monitoring system (SA Instruments Inc., Stony Brook NY).

Lung fibrosis imaging protocol: Images were acquired with the following sequences and parameters: 3D Ultra Short TE (UTE) sequence with respiratory gating (Matrix 192 x 192; TR/TE/flip angle = 8ms/0.02ms/40°; field of view (FOV) 48 x 48 mm; one average; slice thickness=250μm, acquisition time=30 minutes, spatial resolution=250μm isotropic); 3D Fast Low Angle Shot (FLASH) (Matrix 96 x 192; TR/TE/FA=15ms/1.5ms/40°; field of view (FOV) 24 x 48 mm; one average; slice thickness=250μm, acquisition time=2-3 minutes, spatial resolution=250μm isotropic); T1-weighted Rapid Acquisition with Relaxation Enhancement (RARE) (Matrix 192 x 192; TR/TE=1000ms/26ms; field of view (FOV) 33 x 33 mm; one average; slice thickness=1mm, acquisition time=2 minutes, spatial resolution=172μm). After a localizer image to position the animal, RARE images were acquired in coronal and axial orientations to provide images with good anatomical contrast. This was followed by baseline FLASH and UTE images. The probe was delivered as a bolus via an indwelling tail vein catheter. Immediately after injection the FLASH sequence was repeated 5 times to demonstrate probe injection and to measure clearance of probe from the blood and other organs. At 12 min post-injection, the UTE image was repeated.

Lung image representation: A subtraction image was generated in Osirix (Geneva, Switzerland) by subtracting the UTE pre probe injection image from the UTE post probe injection image. A mask was applied to the lung based on the anatomical scan. The resulting difference image was then false colored, and superimposed on the corresponding anatomical scan image.

Lung image analysis: We first visualized the vasculature on the anatomical coronal images (T1-weighted RARE sequence) to delineate lung parenchyma from vessels and airways to define the regions of interest (ROI). The FLASH image immediately post injection was also used to identify large vessels. One ROI, excluding large vessels and airways, was placed over the left lung and one ROI was placed over the right lobes of the lung; additional ROIs were placed over the left forearm muscle and the right forearm muscle, while seven ROIs were placed in the field of view without any tissue (air) to measure the variation in background signal. These ROIs were then copied onto the UTE images to quantify signal intensity (SI). Coronal slices that cover the entire lung were analyzed (>10 250 μm slices per mouse). The lung ROI size ranged from 0.7-45.6 mm^2 with 44-68 ROIs used to cover the entire lung. The analysis was repeated for the pre-probe and post-probe UTE data sets. Image visualization and quantification was performed in Osirix.

To quantify signal enhancement in the lung, contrast to noise ratio (CNR) was calculated using equation S2 where SI=signal intensity, SD=standard deviation. An average of all image slices was calculated for the pre-injection images (CNR_{pre}) and for the post-injection images (CNR_{pos}). The lung enhancement for each mouse is expressed as ΔCNR, the difference between the pre-injection CNR and the post-injection CNR (equation S3).

$$CNR = (SI_{lung} - SI_{muscle})/SD_{air} \qquad (S2)$$
$$\Delta CNR = CNR_{post} - CNR_{pre} \qquad (S3)$$

Liver fibrosis imaging protocol: Images were acquired with the following sequences and parameters: 3D FLASH with respiratory gating (Matrix 128 x 64; TR/TE/FA=11ms/1.4ms/10°; FOV 24 x 24 mm; one average); T1-weighted RARE (Matrix 64 x 140; TR/TE=3700ms/7.7ms; field of view (FOV) 49 x 22.5 mm; six averages). After a localizer image to position the animal, RARE images were acquired in axial orientations to provide images with good anatomical contrast. This was followed by the FLASH sequence at baseline, immediately after i.v. bolus probe injection, and again at 15 minutes post-probe injection.

Liver imaging analysis: A region of interest (ROI) was manually traced encompassing the liver parenchyma while avoiding major blood vessels. A second ROI was placed on the dorsal muscle visible in the same image slice to quantify the signal intensity in the muscle for comparison. Seven ROIs were placed in the field of view without any tissue (air) to measure the variation in background signal. We analyzed more than 20 axial slices per mouse across the entire liver in this fashion. The same analysis was performed on the pre- and 15-minute post injection images acquired with the FLASH sequence. Image visualization and quantification was performed in Osirix. CNR was similarly calculated by normalizing to the signal intensity in the muscle with eq S2. ΔCNR was similarly calculated by subtracting the CNR_{Pre} from CNR_{Post}, eq S3.

Tissue analysis

To assess pulmonary fibrosis, the left lung was homogenized and analyzed for allysine, LOX activity, and hydroxyproline content. The right lung lobes were subject to hematoxylin and eosin (H&E) staining, Sirius red staining, and qRT-PCR analysis.

To assess hepatic fibrosis, liver was harvested and analyzed for hydroxyproline, H&E staining, Sirius red staining, and qRT-PCR analysis.

Hydroxyproline assay: In a high-pressure reaction tube containing 250 μL lung homogenate was added 150 μL water, 100 μL 4 mM sarcosine as an internal standard, and 500 μL 12M HCl. The reaction vessel was capped with a teflon cap and heated at 110 ^{o}C for 24h. After hydrolysis the reaction solution was cooled to r.t. and neutralized with 6M NaOH. To 100 µL of the supernatant was added 900 µL borate buffer (0.7M boric acid, pH 9.5) in a glass tube. Next 100 µL of *o*-phthaladehyde (50 mg) dissolved in 1 ml acetonitrile containing 26 µL of β-mercaptoethanol was added, followed 60 s later by 100 µL of iodoacetamide reagent (140 mg/ml of iodoacetamide in acetonitrile). One minute later, 300 µL of 5mM FMOC in acetone was added with vortexing. Five minutes after FMOC addition, 3 mL of ethyl ether was added and the reaction vortexed. The organic layer was discarded and the wash repeated. The aqueous phase was then diluted with 5 mL water. Hydroxyproline standards were prepared with hydroxyproline:sarcosine ratios of 1:4, 1:2, 1:1, 2.5:1 and 5:1 at a constant concentration of sarcosine of 100 µM. Standards and tissue samples were then subject to HPLC analysis. The hydroxyproline:sarcosine peak ratio was used to quantify hydroxyproline concentration.

Allysine assay: The allysine concentration in murine lung tissue samples was quantified using an adaption of the p-cresol protocol described by Umeda et al (33). *p*-Cresol was replaced with fluorescent sodium 2-naphthol-7-sulfonate to increase the sensitivity of detection. In a high-pressure reaction tube containing 250 µL lung homogenate was added 200 µL water, 50 µL 4 mM fluorescein as an internal standard, 500 µL 12M HCl and 20 mg of sodium 2-naphthol-6-sulfonate (TCI America). The reaction vessel was capped with a teflon cap and heated at 110 ^{o}C for 24h. The reaction solution was cooled, neutralized with 6M NaOH and analyzed by HPLC. HPLC conditions: Solvent A: H_2O + 0.1% TFA, Solvent B: MeCN + 0.1% TFA. 0-40min; 5-30% solvent B, 40-42mins; 30-95% solvent B, 42-45mins; 95% solvent B, 45-47mins; 95-5% solvent B, 47-50mins; 5% solvent B. Column: C8 Phenomenex 25mm x 4 mm, 5µm. 0-40mins λ_{ex} = 254, λ_{em} = 310nm, 40-50mins λ_{ex} = 490nm, λ_{em} = 510nm.

Peak areas were corrected according to a fluorescein standard (elution time: 45mins) and allysine concentration in nmol/Lung calculated using a standard curve (plot of concentration of the pure bis-naphthol derivative of allysine vs. HPLC peak area), and a correction for lung mass.

LOX assay protocol: Enzymatic activity measurement was based on the production of H_2O_2 as previously described (15, 35). Briefly, LOX was first extracted by adding protease inhibitors (PI) (2X, PBS buffer) (0.25 mL) to 0.25 mL of freshly prepared lung homogenate, and left for 4h at 4°C on a bench shaker. Lung homogenate was then spun down (13,000 rpm, 20 mins, 4°C), supernatant removed, and the pellet washed twice with PBS buffer. The remaining pellet was re-suspended in 4M Urea, 50mM sodium borate buffer, pH 8.2 buffer, and left overnight at 4°C on a bench shaker. Lung homogenate was spun down (13,000 rpm, 20 mins, 4°C), the supernatant underwent buffer exchange and concentrated at 4°C using a 10k MWCO centrifugal filter (Millipore) to give a final volume of 300 µL in 1.2M urea, 50 mM sodium borate buffer, pH 8.2.

LOX activity in each lung sample was then determined by fluorescence assay. Briefly, each sample was incubated with 0.5 mM of pargyline, to inhibit any potential endogenous monoamine oxidase A and B. In a 96 well plate (Greiner 96 Well Black Microclear Base), 100 µL of tissue homogenate was incubated with 20 µL of pargyline for 1h at 37 °C with or without the pan-LOX inhibitor, BAPN (100 µM). A reaction mixture containing Amplex Red (125 µM- for a final concentration 50µM), horseradish peroxidase (2.5 U/ml- for a final concentration 1U/ml) and diaminopentane (37.5 µM – for a final concentration 15 µM) was prepared in 1.2 M urea, 50 mM sodium borate buffer, pH 8.2. 80 µL of the reaction mixture was added into each well after the 1h incubation. The relative fluorescence units (RFU) were read every 1 min for 60 min at 37°C, excitation 565 nm and emission 590 nm, and the slope of the kinetic curves for each sample in the linear range was calculated using PRISM data analysis software. The difference between the signals obtained in the presence of the BAPN inhibitor and in the absence of the inhibitor was considered to be the specific total LOX activity in the sample. Slopes of the data in RFU/min were converted to pmol H_2O_2/min/Lung using a standard curve of [H_2O_2] vs. RFU and a correction for lung mass.

Histology: Tissue harvested was fixed in neutral 10% buffered formalin, embedded in paraffin, and sectioned into 5 µm-thick slices for hematoxylin and eosin (H&E), Sirius red/fast green staining, and Verhoeff's elastin staining. Sirius red/fast green and elastin stained slides were scanned and images were digitally acquired. Images were analyzed by an experienced pathologist to classify the amount of fibrosis in a blinded fashion, and based on a comprehensive scoring system. To assess fibrotic lung disease severity, Ashcroft score was assigned to each mouse analyzed (28). Liver disease was staged according to Ishak score (36). In addition, Sirius red stained slide images were analyzed by thresholding to determine the collagen proportional area (CPA), % area stained with Sirius Red collagen positive, using Image J (National Institute of Health, Bethesda MD) as previously described (16-18, 20).

mRNA isolation and qRT-PCR: Lung and liver tissue were harvested and snap frozen in liquid nitrogen. RNA was extracted with TRIzol reagent (Thermofisher) according to the manufacturer's instructions. Total RNA from each sample was treated with DNase I (Promega), and subsequently used to create cDNA by single strand reverse transcription with the SuperScript III First-Strand Synthesis SuperMix (Thermofisher). Gene expression analysis was completed using commercial TaqMan primers (Lox: Mm00495386_m1; Loxl1: Mm01145738_m1; Loxl2: Mm00804740_m1, Loxl3: Mm01184865_m1; Loxl4: Mm00446385_m1; Sma: Mm00725412_s1; Col1a1: Mm00801666_g1; all from Thermofisher) on an 7900HT Real Time PCR system. 18s ribosomal subunit RNA served as the endogenous control. Gene expression was calculated using the ΔΔCt method. All reactions were performed in duplicate and repeated to ensure reproducible results.

Supplemental References

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Collagen crosslinking reaction schematics:

Scheme S3: Collagen crosslink species reactions and chemical structures.

Supplemental figures:

Supplemental Figure 1: Probe purity characterization of Gd-Hyd by HPLC-ICP-MS. Spectrum shows only a single Gd species with no free Gd present ($R_t = 2.3$mins). R_t (6.8 mins) correlates with LC-MS peak of correct mass ion for Gd-Hyd.

Supplemental Figure 2: HPLC-ICP-MS purity analysis of Gd-DiMe. Spectrum shows only a single Gd species with no free Gd present. R_t (9.2 mins) correlates with LCMS peak of correct mass ion for Gd-DiMe.

Supplemental Figure 3: Relaxivity, r_1, of Gd-Hyd (**A**) and Gd-DiMe (**B**) measured in PBS buffer, pH 7, 37 °C at 1.4T, in the absence ('PBS') or presence of Bovine Serum Albumin ('BSA'), oxidized BSA ('BSA-ALD'), or oxidized BSA after addition of cyanoborohydride ('BSA-ALD + CNBH3'). An increase in relaxivity in the presence of protein is indicative of protein binding due to the increased rotational correlation time of the chelate-protein complex. No significant increase in relaxivity was observed going from Gd-Hyd in PBS alone (4.07 $mM^{-1}s^{-1}$) to Gd-Hyd in BSA solution (4.17 $mM^{-1}s^{-1}$). However the relaxivity measured in BSA-ALD (4.57 $mM^{-1}s^{-1}$, 12.3% increase) was significantly higher for Gd-Hyd in PBS alone (P=0.038). Addition of $NaCNBH_3$ to reduce the hydrazone and make the Gd-Hyd bond to BSA-ALD irreversible resulted in a 37.3% increase in relaxivity relative to Gd-Hyd alone (r_1 = 5.59 $mM^{-1}s^{-1}$, P=0.0021). No significant difference in relaxivity was seen for the Gd-DiMe probe for either BSA (4.20 $mM^{-1}s^{-1}$) or BSA-ALD (4.22 $mM^{-1}s^{-1}$) samples with respect to Gd-DiMe measured in PBS alone (4.09 $mM^{-1}s^{-1}$).

Supplemental Figure 4: After the relaxivity experiments (Supplemental Figure 3), the solutions were subjected to ultrafiltration across a 5 kDa cutoff membrane. The concentration of unbound Gd probe in the filtrate was quantified by ICP-MS to determine the amount of probe bound to protein. Panel (**A**) shows nmol of Gd-Hyd (or Gd-DiMe) associated with protein. A ten-fold increase in Gd-Hyd probe bound to protein was observed for ALD-BSA (2.52 nmol, 5.72% of total [Gd]) compared to BSA (0.24 nmol 0.58% of total [Gd]). The amount of Gd-Hyd probe bound to ALD-BSA increased to 6.78 nmol (16.63% of total [Gd]) upon the addition of $NaCNBH_3$ reducing agent. No significant amount of Gd-DiMe was found bound to BSA or ALD-BSA. (**B**) The relaxivity of the ALD-BSA bound complex is calculated from the amount of Gd-Hyd bound to the protein in panel (**A**) and the relaxivity measured in Supplemental Figure 3. The relaxivity of the protein bound Gd-Hyd is 13.74 $mM^{-1}s^{-1}$, increases by 3.4 fold compared to a relaxivity of 4.07 $mM^{-1}s^{-1}$ when measured in PBS alone. *$p<0.05$, **$p<0.01$, ****$p<0.0001$, ns=not significant, Gd-Hyd samples in (A) was analyzed by ANOVA, Gd-DiMe and protein bound vs. free solution were analyzed by t-test.

Direct binding of Gd-Hyd to oxidized collagen could not be performed due to an inability to generate high concentrations of allysine on the insoluble collagen protein. Instead, we used a soluble assay that involved oxidizing the lysine residues on BSA to allysine. When incubated with ALD-BSA, Gd-Hyd shows an increased binding to the protein compared to normal BSA. This increase in protein binding translates to an increase in relaxivity compared to the Gd-Hyd in solution as a result of immobilization of the small molecule on the surface of a large biomolecule. No such increase in relaxivity was observed with BSA or for the Gd-DiMe control probe, suggesting that a non-specific interaction with the protein was not responsible for the relaxivity enhancement. The specificity of the interaction was confirmed by addition of cyanoborohydride, which reduced the hydrazone to an irreversible hydrazide linkage with the protein. The Gd-Hyd:aldehyde interaction with ALD-BSA would be analogous to that possible with the allysine functionality present on collagen.

Supplemental Figure 5: T1 transmetallation stability study. Evolution of the relative water proton paramagnetic longitudinal relaxation rate $R_1(t)/R_1(0)$ vs. time for Gd-Hyd and Magnevist (Gd-DTPA) when challenged with Zn^{2+} and phosphate at pH 7, 37 °C. A decrease in $R_1(t)/R_1(0)$ is indicative of Gd^{3+} release from the complex and precipitation as the insoluble phosphate salt. Transmetallation of Gd-DTPA under these conditions is consistent with literature. There is no evidence of transmetallation for the DOTA-based, macrocyclic Gd-Hyd complex, in keeping with the high kinetic inertia and thermodynamic stability of macrocyclic gadolinium chelates.

Supplemental Figure 6: Serum stability study. (**A**) Overlay of HPLC-ICP-MS traces of Gd-Hyd sampled at regular time intervals after incubation in rat serum. (**B**) Quantification of data in (**A**) expressed as % of intact Gd-Hyd present in rat serum at each time point. Gd-Hyd incubated in rat serum at 37^{o}C is completely stable up to 8 hours. Gd-Hyd stability is >95% after 24 hours in serum. There is no evidence of dechelated (“free”) gadolinium, which has a different retention time from Gd-Hyd or its degradation products.

Organ	%ID per Gram	%ID per Organ
Serum	0.00393±0.000879	0.00556±0.000441
Brain	0.00409±0.000421	0.00156±0.000103
Lung	0.0357±0.00963	0.00401±0.000554
Liver	0.0650±0.0126	0.0674±0.00855
Muscle	0.00917±0.00217	0.0855±0.00783
Heart	0.0261±0.00880	0.00275±0.00118
Spleen	0.0929±0.0695	0.00689±0.00586
Intestine	0.0205±0.00267	0.0304±0.00345
Stomach	0.0282±0.00560	0.00840±0.000816
Bone	0.0208±0.00348	0.0245±0.00231
Kidneys	0.708±0.223	0.205±0.0216
Tail	0.0408±0.0116	N/A

Supplemental Table 1: Gd-Hyd is readily cleared from the body 24 hours after bolus i.v. injection. Biodistribution of Gd-Hyd at 24 hours after bolus intravenous injection in C57Bl/6 mice showed that almost all of the gadolinium (>99.5%) has been eliminated from the body after 24 hours. The mean ± 1 standard deviation for each organ is tabulated. ID=Injected Dose.

Supplemental Figure 7: mRNA expression for lysyl oxidase and its paralogs in the mouse lung. (**A-D**) Loxl3 expression was significantly elevated 2 weeks after bleomycin instillation. Loxl3 expression significantly increased from 1 to 2 week post-bleomycin challenge. BAPN-treated bleomycin mice had significantly increased Lox, Loxl1, Loxl3, and Loxl4 expression, compared to sham (PBS treated) control mice. In each case, no reduction was seen compared to the 2-week bleomycin animals. *p<0.05 compared to sham, unless otherwise noted, ANOVA.

Supplemental Figure 8: BAPN treatment does not impact α-Sma or Collagen I mRNA expression in 2 week post-bleomycin mice. Bleomycin-induced fibrosis increased α-Smooth muscle actin (Sma) in (**A**), and Collage, Type 1, alpha 1 (Col1a1) mRNA expression in (**B**). Similarly elevated Sma and Col levels were seen in bleomycin mice with or without BAPN treatment.

Supplemental Figure 9: Accumulation of elastic fibers in bleomycin-injured mouse lungs. (**A**) Verhoeff's elastin stained lung histological sections of mice after instillation of PBS (sham), 1 unit/kg of bleomycin for 1 week (1wk Bleo, *n=5*), 2 weeks (2wk Bleo, *n=5*), and 4 weeks (4 wk Bleo *n=7*). (**B**) Quantification of elastin positive area. Scale bar=100µm. $^*p<0.05$, $^{***}p<0.001$, ANOVA.

Supplemental Figure 10 Gd-Hyd imaging of liver fibrosis is specific. Gd-Hyd liver enhancement was significantly higher in fibrotic mice treated with CCl_4 for 12 weeks compared to mice treated with vehicle. No significant liver enhancement was seen with Gd-DiMe, suggesting specificity. ***p<0.001, ns=not significant, ANOVA.

Supplemental Figure 11: mRNA expression of lysyl oxidase and its paralogs in the liver after CCl_4 treatment. (**A-B**) Lox and Loxl3 expression were significantly elevated at 6 weeks (*n=7-10*) and 12 weeks (*n=7*) after CCl_4 treatment compared to vehicle treated animals (Veh, *n=15*), and significantly reduced after 6 weeks of withdrawal (WD, *n=6*) compared to the 12-week CCl_4 cohort. (**C**) Loxl4 expression was significantly elevated in the 6 week CCl_4 cohort (*n=7*) compared to vehicle treated controls (*n=15*), but not in the 12 week CCl_4 (*n=7*) or the withdrawal (*n=6*) groups. *$p<0.05$, ****$p<0.0001$, ANOVA.